

Mass spectrometry based protein identification with accurate statistical significance assignment

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Received on XXXXX; revised on XXXXX; accepted on XXXXX

Associate Editor: XXXXXXXX

ABSTRACT

Motivation: Assigning statistical significance accurately has become increasingly important as meta data of many types, often assembled in hierarchies, are constructed and combined for further biological analyses. Statistical inaccuracy of meta data at any level may propagate to downstream analyses, undermining the validity of scientific conclusions thus drawn. From the perspective of mass spectrometry based proteomics, even though accurate statistics for peptide identification can now be achieved, accurate protein level statistics remain challenging.

Results: We have constructed a protein ID method that combines peptide evidences of a candidate protein based on a rigorous formula derived earlier; in this formula the database P -value of every peptide is weighted, prior to the final combination, according to the number of proteins it maps to. We have also shown that this protein ID method provides accurate protein level E -value, eliminating the need of using empirical post-processing methods for type-I error control. Using a known protein mixture, we find that this protein ID method, when combined with the Sorić formula, yields accurate values for the proportion of false discoveries. In terms of retrieval efficacy, the results from our method are comparable with other methods tested.

Availability: The source code, implemented in C++ on a linux system, is available for download at ftp://ftp.ncbi.nlm.nih.gov/pub/qmbp/qmbp_ms/RAID/RAID.Linux.64Bit

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1 INTRODUCTION

Peptide identifications (ID) via mass spectrometry (MS) have become the central component in modern proteomics; this component, combined with additional analyses, routinely yields pragmatic meta data, including protein ID, protein quantification, protein structure and protein associations (Zhang *et al.*, 2013). These meta data, especially the associated statistical significance assignments, need to be as accurate as possible because they often form the building blocks for investigations at the systems biology level and influence the scientific conclusions drawn henceforth. In this paper, we focus on protein ID, in particular on improving the accuracy of statistical significance assigned to proteins identified.

The need for robust developments towards accurate statistical significance assignments has been advocated (Noble and MacCoss, 2012; Huang *et al.*, 2012) despite the existence of many protein ID methods (McHugh and Arthur, 2008; Serang and Noble, 2012;

Li and Radivojac, 2012). It has also been suggested (Spirin *et al.*, 2011) that the primary cause of unreliable significance assignment for protein ID can be attributed to inaccurate significance assignment for peptide ID. Frequently used error-control/significance-assigning methods for peptide ID largely fall into two groups: proportion of false discovery (PFD), which is often incorrectly termed as false discovery rate (Benjamini and Hochberg, 1995), and spectrum-specific P -value/ E -value (Fenyo and Beavis, 2003; Alves *et al.*, 2007; Park *et al.*, 2008). Methods belonging to the first group, controlling type-I error globally only, do not discriminate among identified peptides (Elias and Gygi, 2007). Methods belonging to the second group, capable of assigning per-spectrum per-peptide significance, can properly prioritize identified peptides when reported P -/ E -values are accurate; but the needed statistical accuracy is often unattainable due to improper heuristics or unjustifiable distribution assumptions (Alves *et al.*, 2007; Segal, 2008; Spirin *et al.*, 2011).

Given a tandem MS (MS/MS) spectrum and a quality score cutoff S_c , the E -value $E(S_c)$ should reflect the expected number of random peptides with scores the same as or better than S_c . (Similarly, the P -value $P(S_c)$ reflects the probability of finding a random peptide with quality score $S \geq S_c$.) In general, the E -value is obtained by multiplying the P -value by the total number of *qualified peptides* (whose masses fall in the range $[m_p - \delta, m_p + \delta]$ with m_p being the precursor ion's mass and δ the specified tolerance) in the database searched. Thus, besides providing the user with the numbers of false positives to anticipate, accurate E -value assignments enable ranking of candidate peptides across different spectra and experiments. In database searches in proteomics, the goal of accurate statistics can be approached in at least two ways. First, one may devise a scoring function whose resulting score distribution can be analytically characterized and thus used to infer the statistical significance (Alves *et al.*, 2007); if this is done correctly, the theoretical score distribution should fit well the bulk part of the normalized score histogram obtained from scoring all *qualified peptides* in the database of interest. Second, one may infer the spectrum-specific P -value via the normalized score histogram obtained from scoring all possible peptides (APP) (Alves and Yu, 2008); in this case, the database dependence appears only in the E -value, which is the P -value multiplied by the number of qualified peptides associated with the specified precursor ion mass and mass error tolerance. Either way yields database-specific E -values. Once a peptide E -value is obtained, one may transform it into the peptide database P -value (DPV) (Yu *et al.*, 2006; Alves *et al.*, 2008b),

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representing the likelihood of obtaining, in the database chosen, at least one peptide scoring equal to or better than the prescribed threshold. When combining P -values of peptides associated with a candidate protein, we use the peptides' DPVs.

Specifically, our proposed protein ID method combines peptide evidences of a candidate protein using a rigorous formula derived earlier (Alves and Yu, 2011); in this formula the DPV of every peptide is weighted, prior to the final combination, according to the number of proteins it maps to. Among the existing protein ID methods, the approach taken by Spirin *et al.* (2011) is closest to ours; both methods combine peptides' spectrum-specific P -values. There are, however, major differences between our method and that of Spirin *et al.* (2011). First, in our method, each candidate peptide of a query spectrum receives a DPV, allowing multiple matching peptides per spectrum. This is to take into account the possibility of peptide co-elution (Alves *et al.*, 2008a). For the method of Spirin *et al.* (2011), only the best peptide match per spectrum is considered and the peptide DPV thus represents the probability of having the *best* match score no worse than the prescribed threshold when searching a database. Since each random protein database only contributes one best match score, searching *many* random protein databases is required for the P -value assignment. Second, the candidate peptides' P -values are combined differently. Our method, down-weighting contributions of peptides mappable to multiple proteins, combines peptide DPVs directly using a rigorous formula (Alves and Yu, 2011); the method of Spirin *et al.* (2011) first transforms, for every candidate protein, the P -values of its associated peptides into Z -scores, combines them using Stouffer's formula (Whitlock, 2005), and then transforms the combined Z -score back to a final P -value with multiple hypotheses testing correction. Third, the cutoff conditions for peptides' P -values are different. Our method approximates DPVs (Yu *et al.*, 2006; Alves *et al.*, 2008b) by E -values, valid for small E -values, and retains all peptides whose E -values are less than one. That is, we have a global cutoff condition. For the method of Spirin *et al.* (2011), the peptide cutoff P -value varies by candidate protein: given a candidate protein, its corresponding peptides' Z -scores are first sorted in descending order; the k th Z -score is chosen as the cutoff provided that the maximum combined Z -score is reached while combining the top k Z -scores using the Stouffer's formula.

There exist many other protein ID methods, for example, ProFound (Zhang and Chait, 2000), ProteinProphet (Nesvizhskii *et al.*, 2003), DBParser (Yang *et al.*, 2004), EBP (Price *et al.*, 2007), PANORAMICS (Feng *et al.*, 2007), PROVALT (McHugh and Arthur, 2008), X!Tandem (Fenyó *et al.*, 2010), Scaffold (Searle, 2010) and npCI (Serang *et al.*, 2013), to name just a few. We refer the readers to recent review papers (Huang *et al.*, 2012; Serang and Noble, 2012) for details and more comprehensive listings of these methods. Although some of them do start with spectrum-specific peptide P -values, they often *assume* certain parametric forms for the peptide score distributions when searching a random database; other methods, however, only process outputs of specific peptide identification tools, limiting their uses to certain platforms. By discarding all but the *best* few peptide scores per spectrum per database search, the method of Spirin *et al.* (2011) does not rely on the accuracy of the full peptide score distribution from searching a random database and in principle can accept input from various peptide identification tools. Our method is free from

the aforementioned problems for different reasons. Founded on a derived analytical formula, our method can be applied in general and will yield accurate protein P -values if the input peptide DPVs (or E -values) are accurate. When using peptide E -values reported by RAId_DbS, even though the parameters of the score distribution are determined via maximum-likelihood, the functional form of the score distribution is analytically derived (Alves *et al.*, 2007) rather than assumed. When the statistical significances are obtained from RAId_aPS (Alves *et al.*, 2010), for every scoring function implemented, the P -values are inferred by scoring APP instead of assuming that the score histogram follows a specific form; the peptide E -values are then obtained via multiplying the P -values by the respective numbers of qualified peptides.

The paper is organized as follows. The mathematical underpinnings of our formalism will be described in the methods section. In the results section, comparisons of our method with other approaches will be made; the accuracy of the reported protein P -value will be illustrated. Some technical but important issues will be addressed in the discussion section. To keep the paper focused, we relegate to supplementary information figures and tables that complement or corroborate the information contained in the main text.

2 METHODS

2.1 Statistical Protocols

Weighting the contribution of each peptide in protein identification is important. It helps mitigate the issue of peptide degeneracy, where an identified peptide is a subsequence of multiple database proteins. The optimal weighting scheme, however, can depend on the protein ID methodology employed. For the purpose of our study, namely, devising a method that yields accurate protein P -values, we opt for a simple weighting scheme: a peptide's weight is inversely proportional to the number of database proteins it maps to. Within a sample, when multiple spectral searches identify the same peptide but with different significance levels, only the most significant assignment of that peptide is retained for further analyses.

The foundation of our method is built upon a rigorous formula (Mathai, 1983; Alves and Yu, 2011) that enables weighted combination of P -values. When the weights are all identical, this formula reduces to Fisher's formula (Fisher, 1932; Bahurucha-Reid, 1960); when the weights are all different, this formula reduces to the formula of Good (Good, 1955). A detailed derivation and generalization to incorporating nearly identical weights can be found in (Alves and Yu, 2011), whose notation will be used to briefly summarize the content of the formula.

Let us assume that a given protein contains L identified peptides with P -values. Let us further group these L peptides, according to the number of database proteins a peptide maps to, into m groups with $1 \leq m \leq L$. Within each group k , the n_k peptide P -values are weighted equally; while peptide P -values in different groups are weighted differently.

The weighting enters our formalism through the following quantities of interest

$$\tau \equiv \prod_{k=1}^m \left[\prod_{j=1}^{n_k} p_{k;j} \right]^{w_k}, \quad (1)$$

$$Q \equiv \prod_{k=1}^m \left[\prod_{j=1}^{n_k} x_{k;j} \right]^{w_k}, \quad (2)$$

where each $p_{k;j}$ represents a reported peptide P -value, each $x_{k;j}$ represents a random variable drawn from an uniform, independent distribution over $(0, 1]$ and each w_k is a positive weight. The quantity of interest $\text{Prob}(Q \leq$

τ), representing the protein P -value, was obtained earlier (Alves and Yu, 2011) and is repeated below for clarity.

Let $F(\tau) \equiv \text{Prob}(Q \leq \tau)$, one may show that

$$F(\tau) = \left[\prod_{l=1}^m r_l^{n_l} \right] \sum_{k=1}^m \sum_{\mathcal{G}(k)} \left\{ \frac{1}{r_k^{g_k+1}} H(-r_k \ln \tau, g_k) \times \right. \\ \left. \times \left(\prod_{j=1, j \neq k}^m \frac{(n_j - 1 + g_j)!}{(n_j - 1)! g_j!} \frac{(-1)^{g_j}}{(r_j - r_k)^{n_j + g_j}} \right) \right\}, \quad (3)$$

where $r_k \equiv 1/w_k$ is the number of proteins a group- k peptide maps to, $\sum_{\mathcal{G}(k)}$ enumerates each set of nonnegative integers $\{g_1, g_2, \dots, g_m\}$ that satisfies the k -dependent constraint $\sum_{i=1}^m g_i = n_k - 1$, and the function H is defined as

$$H(x, n) \equiv e^{-x} \sum_{k=0}^n \frac{x^k}{k!}. \quad (4)$$

See the supplementary information for an example application of formula (3).

When searching a database with a prescribed peptide mass error tolerance δ , one often needs to score different numbers of database peptides for spectra with different precursor ion masses. That is, the number of tested hypotheses (database peptides in the mass range $[m_p - \delta, m_p + \delta]$) varies by the precursor ion mass m_p . The effect of varying number of multiple hypotheses tested can be properly accounted for by using the peptide DPVs (Yu *et al.*, 2006; Alves *et al.*, 2008b) for P -values ($p_{k;j}$) in eq. (1); given a quality score cutoff S_c , the peptide DPV is defined as

$$P_{\text{db}}(S_c) = 1 - e^{-E(S_c)}, \quad (5)$$

where $E(S_c)$ represents the expected number of peptides having score $S \geq S_c$, and the DPV $P_{\text{db}}(S_c)$ represents the probability of seeing one or more peptides in a given random database with quality scores $S \geq S_c$. Another advantage of using DPV is that as a function of the quality score S , the E -value $E(S)$, determined by the search score histogram per spectrum and the number of qualified peptides (database-dependent), correctly takes into account both the spectrum-specificity and the database-specificity of scoring statistics.

Since the E -value specifies the expected number of random database peptides having scores equal to or better than the given cut-off, a peptide with E -value larger than one is more likely to be a false positive than a true positive. For this reason, when constructing the evidence peptide set for identification of a protein, we only include peptides with E -values less than one. This implies that only peptide DPVs less than $(e-1)/e$ are considered, leading to a combination of truncated P -values. Unfortunately, combining truncated P -values, even though doable, is far more complicated than using eq. (3). However, two observations simplify the matter. First, it is evident from eq. (5) that the DPV approaches the E -value when the E -value is small. Second, we note that confidently identified proteins must contain evidence peptides with high identification confidences (or small E -values). Therefore, for practical uses, we may approximate the DPV by its corresponding E -value. Because only E -values less than one are considered, the approximated DPVs (or simply the E -values) now encompass the full range between zero and one. Consequently, it is unnecessary to combine truncated P -values, and the simple formula (3) becomes applicable. The protein E -value is then obtained via multiplying the protein P -value by a Bonferroni correction factor; in this case, the Bonferroni factor is the number of protein clusters (described below) each having at least one evidence peptide with E -value less than one.

We denote by a protein cluster a group of *entangled* proteins that share a substantial portion of evidence peptides. To avoid exaggerating the number of identified proteins, several existing methods (Huang *et al.*, 2012) report those entangled proteins as one. Adopting the same idea, we implemented this strategy via a transitive approach described below. One first sorts the identified proteins by the number of identified evidence peptides in descending order and using the rank of a protein in the sorted list as that

protein's cluster index. Starting with the first protein as the reference protein, all other lower-ranking proteins sharing at least 95% of evidence peptides with the first protein will have their cluster indexes changed to that of the reference protein. One then moves the reference point (from the first) to the second protein, all other lower-ranking proteins sharing at least 95% of evidence peptides with the reference protein will have their cluster indexes changed to that of the reference protein. The reference point is then moved to the third protein and the process continues till the reference point moves through all proteins in the list. The protein with most significant P -value within a cluster (containing one or more proteins) is called the head of that cluster, the other proteins members of that cluster. An exception to the aforementioned clustering rule, however, is introduced to appropriately emphasize a protein's evidence peptides that are not shared by other proteins. We call evidence peptides of this kind *unique* peptides to a protein. When a protein has a unique evidence peptide with E -value less than 10^{-4} , our method doesn't allow this protein to be a member protein of any cluster.

2.2 MS/MS Datasets

Sixty-three spectral datasets were categorized into four data groups. See supplementary tables (Table S1 to S4) for details. Protein mixtures giving rise to spectral datasets were reduced with iodoacetamide, resulting in the addition of the carbamidomethyl group (57.07 Da) to cystine residues. Each protein mixture was further digested with trypsin. Among these spectral datasets, there are also dataset-specific parameters such as the target database, the maximum number of missed cleavage sites allowed, the precursor-ion mass error tolerance and the product-ion mass error tolerance. The dataset-specific parameters are given in the figure caption to provide more information underlying the generation of the figures.

For brevity, we shall denote the MS/MS spectra obtained from a sample by SN followed by its sample index. For example, SN1 denotes the collection of MS/MS spectra acquired from mixture sample one. The first data group, SN1 through SN15, contained MS/MS spectra from replicates of different dilutions of Sigma49, a protein standard mixture composed of 49 known human proteins. The second data group, SN16 through SN26, was downloaded from the Pacific Northwest National Laboratory and contained spectra from eleven whole-cell-lysate samples of protein mixtures of *Escherichia coli* K-12. The third data group, SN27 through SN30, consisted of spectra from four in-house whole-cell-lysate samples of protein mixtures of *Escherichia coli* K-12. Downloaded from PeptideAtlas database, the fourth data group (SN31 through SN63) was composed of spectra from SDS-PAGE protein fractionation extractions of human lung cells.

2.3 Protein Databases and Random Databases

Because protein mixtures from *Escherichia coli* K-12 and *Homo sapiens* were analyzed using their corresponding MS/MS spectra, protein databases for both organisms were thus required. From UniProt <http://www.uniprot.org/downloads>, we downloaded 4,303 non-redundant protein sequences of *Escherichia coli* K-12. A non-redundant *Homo sapiens* protein database, containing 31,236 protein sequences, was obtained from the NCBI site ftp://ftp.ncbi.nlm.nih.gov/refseq/H_sapiens/mRNA_Prot/.

When analyzing statistical significance, it is often required to have random (decoy) databases in addition to the organismal (target) databases. One common problem when using random databases is that for a given precursor ion mass the numbers of qualified peptides in the random database and in the organismal database may significantly differ. This causes an additional uncertainty in assessing statistical significance (Elias and Gygi, 2007; Wang *et al.*, 2009). We can avoid this problem by ensuring that the numbers of qualified peptides per spectrum are identical for both the random and the organismal databases: for each qualified peptide in the organismal database, we generate a corresponding random peptide by randomly shuffling its amino acids.

3 RESULTS

The results will be described in the following order. First, we illustrate that our E -value assignments are accurate at both the peptide and the protein levels. We further show that using the formula proposed by Sorić (1989), our reported PFDs agree well with the target-decoy PFDs. Second, our protein E -value accuracy is compared with that of using the formulas in (Spirin *et al.*, 2011). By extending the formula of Sorić for the method of Spirin *et al.* (2011), we also evaluate the agreement between their reported PFDs and the target-decoy PFDs. Benchmarking with some of the existing protein ID methods will be described in the third part.

3.1 E -value accuracy

The input peptide DPVs for our protein ID method are obtained via eq. (5) using the E -values reported by RAId_DbS. For this reason, the input peptide DPVs (for protein ID) are synonymous with the reported peptide DPVs (from RAId_DbS). As mentioned earlier, the statistical accuracy of our protein ID method relies on the DPVs for the evidence peptides being accurate. We therefore start by comparing the input peptide DPV with its definition. In panel A of Figure 1, the abscissa records the peptide DPV, while the ordinate displays the *observed* DPV (*i.e.*, fraction of spectra having at least one or more matching peptides with reported DPVs smaller than the specified threshold). The agreement between the observed DPV and the reported DPV indicates that the peptide DPVs used as input for our protein ID method are accurate.

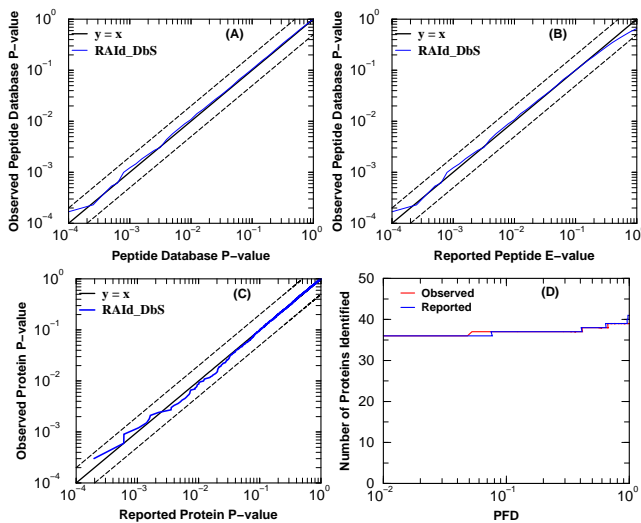


Fig. 1. Assessment of E -value accuracy. In panels A, B, and C, the closer the displayed curves are to the $y = x$ line the better. In panel D, the closer the two displayed curves are to each other the better. See section 3.1 for more details. For panels A, B, and C, spectral dataset SN 26 (*Escherichia coli* K-12 whole cell lysate) is used to search the *Escherichia coli* database with mass accuracy ± 0.033 Da. for precursor and product ions. For panel D, spectral datasets SN 13-15 (Sigma49 protein standard mixture) is used to search the *Homo sapiens* database with precursor ion accuracy ± 0.033 Da. and product ion accuracy ± 0.8 Da.

To assess whether approximating peptide DPVs by their corresponding E -values for E -values less than one is reasonable or not, we plot in panel B of Figure 1 the observed peptide DPVs versus E -values. As expected, when E -values are close

to one, there is certain degree of disagreement; while for small E -values, the agreement is excellent. To assess the accuracy of the protein P -values reported by our eq. (3), we compare them with the observed protein P -values. As described in the method section, the reported proteins appear in clusters, each represented by a *head* protein and its P -value. The observed protein P -value is defined as the fraction of identified protein clusters (whose member proteins each containing at least one evidence peptide with E -value less than one) that have reported P -values smaller than a given threshold. As shown in panel C of Figure 1, good agreement between the reported protein P -value and the observed protein P -value is obtained, indicating that our reported protein P -values are accurate. More protein P -value accuracy assessment examples can be found in Supplementary Figures S1, S2, and S3. With an accurate protein P -value, one can also obtain its corresponding protein E -value by multiplying it by the total number of protein clusters. In Supplementary Figure S4, we show that reported protein E -values obtained this way are accurate.

By having accurate protein E -values, one can avoid the uncertainty associated with using a decoy database (Gupta *et al.*, 2011) while estimating the proportion of false discoveries. In panel D of Figure 1, we plot two PFD curves: one is computed using the reported protein E -value to estimate the number of false identifications (hence the PFD), while the other is computed using the observed PFD obtained from known target protein content in the sample (Sigma49). The excellent agreement between the observed PFD and the reported PFD indicates that one should be able to trust the PFD estimated from accurate reported protein E -values. More accuracy assessment examples of the reported PFD can be found in Supplementary Figure S5.

3.2 Comparison with an EVD-based method

Since the method of Spirin *et al.* (2011) is closest to ours, we also implemented their method and compute equivalent quantities for comparison. Following the Supplementary Material of (Spirin *et al.*, 2011), we have implemented 100 random databases each containing 10,000 random amino acid sequences. However, instead of generating sequences of uneven length, we opt for uniform length (each sequence is of length 350) and generate these random sequences using the background amino acid frequencies of Robinson and Robinson (1991). The EVD parameters are obtained by using only the best score per database search and by applying standard procedures described in (Spirin *et al.*, 2011). The effect of database size difference, leading to rescaling of the α parameter, is done the same way as in (Spirin *et al.*, 2011).

A moment of reflection reveals that the best match P -value of (Spirin *et al.*, 2011) is in fact the DPV (Yu *et al.*, 2006; Alves *et al.*, 2008b). We therefore plot in panel A of Figure 2 the reported peptide DPVs against the observed peptide DPVs. The result indicates that the peptide DPV reported by Spirin *et al.* (2011) is quite accurate, with an uncertainty of a factor of 2 as reported by Spirin *et al.* (2011).

To have a fair assessment, the same procedure for clustering proteins is also applied to the proteins identified using protocols of Spirin *et al.* (2011). The observed protein P -value is defined similarly. Database proteins that contain any of the best match peptides, one from each spectrum, form the effective protein set. The observed protein P -value is simply the fraction of proteins in the effective protein set that have reported protein P -values

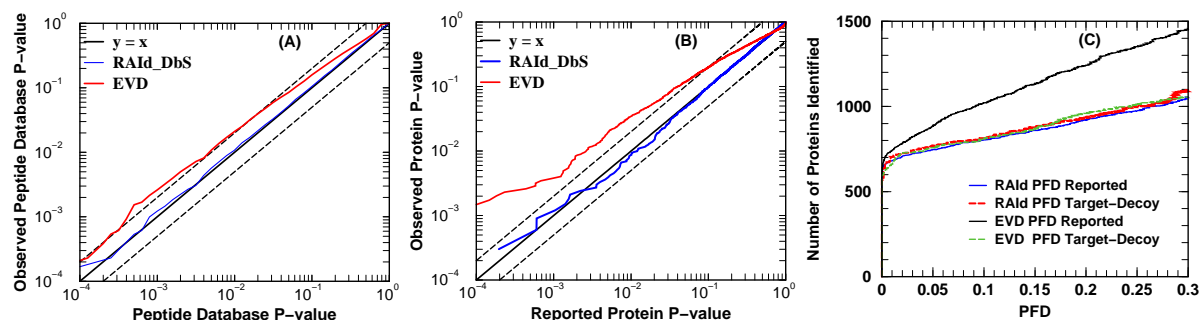


Fig. 2. Statistical Accuracy Comparison. Except that results from two methods are being displayed, panels A, B, and C display similar information respectively to panels A, C and D of Fig. 1. See text in section 3.2 for more details.

less than the specified threshold. The reported protein P -value for the head protein of each cluster is obtained by applying the iterative procedure (involving uses of Stouffer's formula) described in (Spirin *et al.*, 2011). In panel B of Figure 2, the reported protein P -values are plotted against the observed protein P -values. The agreement between the reported protein P -values and the observed protein P -values is not as great as in the peptide case. The protein E -value is then obtained by multiplying the protein P -value by the total number of proteins in the effective protein set.

To construct a PFD curve, it is necessary to estimate the number of false identifications at a given significance threshold. The number of false identifications can be estimated either by using the reported protein E -values or the number of identifications within the decoy databases. The latter is currently widely used mainly because accurate protein E -values (or P -values) are generally hard to attain. To investigate the agreement between the PFD curves obtained using decoy databases and using reasonably accurate protein P -values, we use spectra acquired from dataset SN 26 and construct the PFD curves obtained using both approaches. The good agreement between our E -value based PFD (Sorić, 1989) and the target-decoy based PFD, displayed in panel C of Figure 2, is expected because, as shown in panel D of Figure 1, we have already found that the reported PFD and the observed PFD (computed by using a known protein mixture) are nearly identical. The disagreement between the E -value based PFD and the target-decoy based PFD using protocols of (Spirin *et al.*, 2011) seems to indicate that the moderate uncertainty in DPV can influence the accuracy of the overall PFD estimate in a substantial manner.

For RAId_DbS, the agreement between our E -value based PFD and the target-decoy based PFD is further tested using more spectral datasets (SN16-SN25), see supplementary Figure S6. In addition to RAId score, RAId_aPS allows other scoring functions: XCorr, Hyperscore, and Kscore. For completeness, we plot their corresponding protein P -value accuracy assessments in supplementary Figures S7-S9; we also present the agreement tests between their E -value based PFDs and the target-decoy based PFDs in supplementary Figures S10-S12.

3.3 Comparison with other methods

The previous two subsections focus on the accuracy of type-I error control. Although it is possible to accurately control type-I error for some protein ID methods, this seems not the central focus of all protein ID methods. Many protein ID methods prefer to use the decoy database search results to pragmatically provide statistical

significances for retrieval results from the target (organismal) database. When this approach is used, the retrieval results are displayed in terms of a parametric PFD plot: the parameter is some kind of significance score used to prioritize the identifications, the abscissa shows the proportion of false discovery and the ordinate displays the number of identifications found in the target database. In general, a large number of target identifications at a small PFD value indicates a good retrieval, provided that the number of decoy identifications accurately reflects the number of false identifications in the target database. However, one should note that the fulfillment of the aforementioned condition requires accurate type-I error control. Investigating and improving the statistical accuracy of type-I error control of existing protein ID methods is beyond the scope of this paper and we believe that it is best done by developers of individual protein ID software.

To examine how our method compares with others under the pragmatic target-decoy approach, we analyze two large datasets from *E. Coli* (SN 27-30) and Homo Sapiens (SN 31-63) using a variety of protein ID software along with a number of scoring functions. The list of software is given below (with both software version and scoring functions, if given, shown inside a pair of parentheses): RAId_DbS (v. Jan.12.2014; RAId), RAId_aPS (v. Jan.12.2014; XCorr, Kscore, Hyperscore), Mascot (v. 2.4.0, <http://www.matrixscience.com/help.html>), and X!Tandem (v. 2013.06.15; Hyperscore). The peptide identification software SEQUEST (Eng *et al.*, 1994) (v. 28) is only used in conjunction with other post-processing protein ID software. We list below the post-processing software used (with software version, peptide ID software, and peptide scoring functions, if given, shown inside a pair of parentheses): iProphet (v. TPP 4.5; X!Tandem; Kscore), Proteome Discoverer (v. 1.3, <http://www.thermofisher.com/en/home.html>; SEQUEST, Mascot), and Scaffold Q+/Q+S (v. 4.0, <http://www.proteomesoftware.com>; SEQUEST, Mascot). The results are displayed in different panels of Figure 3. Before delving into the details of the results, we first provide the information relevant to the generation of the results.

In terms of peptide identification, RAId_DbS, RAId_aPS, Mascot, SEQUEST, and X!Tandem used the same parameters: for *Escherichia coli* whole cell lysate, SN 27-30, the precursor ion mass error tolerance is ± 0.033 Da., the product ion mass error tolerance is ± 0.033 Da., and up to 5 missed cleavages are allowed; for *Homo sapiens* heart cells, SN 31-63, the precursor ion mass error tolerance is ± 1.4 Da., the product ion mass error tolerance is ± 0.4 Da., and up to 2 missed cleavages are allowed.

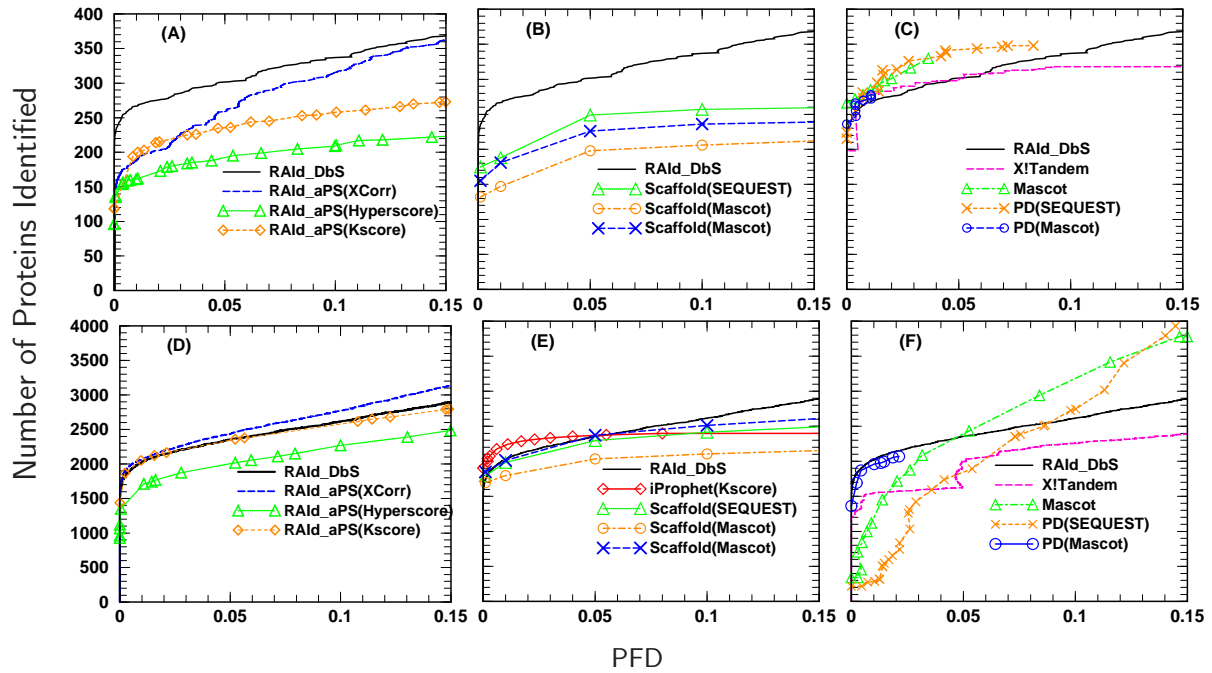


Fig. 3. Retrieval results of various methods based on their stated PFD values. Because we can only ensure the accuracy of type-I error of the proposed method, this figure only illustrates how our retrieval fares at the stated value when compared to other methods. See the text of section 3.3 for more details. To avoid clutter, results from using samples of *Escherichia coli* whole cell lysate, SN 27-30, are displayed in three panels (A, B, and C). Within each panel, the results from RAId_DbS are always shown as a reference curve. Similarly, results from using samples of *Homo sapiens* heart cells, SN 31-63, are also displayed in three panels (D, E, and F). The iProphet (Shteynberg *et al.*, 2011) results in panel E were downloaded from PeptideAtlas instead of being computed.

Both X!Tandem and Mascot have built-in protein ID capability, and the target-decoy approach was directly applied to estimate the protein level PFD. The peptide ID outputs from SEQUEST and Mascot were also further analyzed using Proteome Discoverer for protein identification and the target-decoy approach was applied to estimate PFD. For iProphet, we did not compute the PFD but downloaded the results for data group 4 from PeptideAtlas. Peptide identification in this case was done using X!Tandem (v. 2009.10.01; Kscore).

Whenever the decoy peptide search results are available, Scaffold computes the PFDs using the target-decoy approach; otherwise, it computes the PFDs using a probabilistic method. In Figure 3 three Scaffold PFD curves are displayed, two of which (shown in triangles and circles) are from target-decoy approaches. The protein PFDs under Scaffold were computed by fixing the peptide threshold at 20% PFD with a minimum of 1 evidence peptide per protein. We observed that changing the peptide threshold to lower values had a small effect on the number of proteins identified. We thus used the minimum of 1 peptide per protein to maintain consistency across all methods. For RAId_DbS and RAId_aPS, the PFD estimates do not require user-added target-decoy methods. RAId_DbS and RAId_aPS compute the PFDs using the Sorić formula (Sorić, 1989).

Examinations of different panels of Figure 3 indicates that the retrieval efficacy of the proposed method (shown in RAId_DbS and RAId_aPS PFD curves) is comparable with existing protein ID methods, even though only at the stated values. However, it should be noted that the proposed method does have a few advantages. First, it reports accurate protein P -values, providing accurate type-I error control. Second, the PFD curves obtained using this method show stability across different mass resolution requirement and data

sets, while some methods seem to exhibit fluctuations of notable amplitudes.

4 DISCUSSION

Our investigation indicates that it is possible to achieve faithful protein P -value assignment, hence accurate type-I error control, in protein identifications. Since our approach is founded on a derived mathematical formula that requires accurate peptide E -values as input, it is evident that accurate protein P -values require accurate statistical significance at the peptide identification level.

The discrepancy between the computed protein P -value and the PFD results in our implementation of the method of Spirin *et al.* (2011) is interesting. Based on the results in Fig. 2, the peptide P -values are reasonably accurate albeit exhibiting slightly larger fluctuations than the results from RAId_DbS. In addition to the possibility of accumulating uncertainty of peptides' P -values, the other possibility is that the iterative procedure to choose the combination yielding the most significant Z-score may skew the P -values towards the significant side. Investigation of the origin of the PFD and P -value discrepancy when using the method of Spirin *et al.* (2011), however, is beyond the scope of the current study and might be most appropriately done by the authors of (Spirin *et al.*, 2011).

As explained earlier, we allow more than one candidate peptide per spectrum to accommodate the possibility of peptide co-elution. However, readers may ask why do we choose to use DPVs for lower-ranking peptides per spectrum instead of using ordered statistics. The reason is that in this context using ordered statistics beyond the first is not meaningful: the n th ordered statistics assumes that for a given query spectrum the best $n - 1$ scored peptides

are spurious while the rank- n peptide is the underlying peptide whose fragmentation yields the query spectrum. This contradicts the general idea of using a scoring function: among candidate peptides of a query spectrum, the better a peptide scores the more likely it is the underlying peptide. On the other hand, when using the DPV for the rank- n peptide, we are essentially assuming that the top $n - 1$ candidate peptides of the query spectrum are co-eluted underlying peptides and are not considered to be spurious.

The protein identification method proposed in this paper illustrates the possibility of accurate type-I error control, providing a theoretically sound significance assignment method that is also pragmatically simpler than the target-decoy approach. This is particularly important since the number of identified proteins versus PFDs provides trustworthy retrieval results only if the reported PFDs truly reflects the proportion of false discoveries. Evidently, to achieve accurate type-I error control is a task best done by developers of individual software. Only when this is accomplished can a true retrieval comparison among different methods be done.

Since we did not focus on type-II error, there is definite room for improvement in terms of retrieval efficacy. We note that the information of negatives (segments of a candidate protein not covered by the protein's evidence peptides) is not used. We also believe that, in principle, scoring functions for peptide identification can also be improved to better separate true underlying peptides from false positives. Currently, we are using a flat peptide weight (by the number of proteins a peptide covers). It is perceivable that more sophisticated weighting may be useful in better separating true positive proteins from false positives. It is our plan to investigate these avenues of improvement in the near future.

ACKNOWLEDGEMENT

Funding: This work was supported by the Intramural Research Program of the National Library of Medicine at the National Institutes of Health.

REFERENCES

- Alves, G. and Yu, Y. K. (2008). Statistical Characterization of a 1D Random Potential Problem - with applications in score statistics of MS-based peptide sequencing. *Physica A*, **387**(26), 6538–6544.
- Alves, G. and Yu, Y. K. (2011). Combining independent, weighted P-values: achieving computational stability by a systematic expansion with controllable accuracy. *PLoS ONE*, **6**(8), e22647.
- Alves, G., Ogurtsov, A. Y., and Yu, Y. K. (2007). RAId.DbS: peptide identification using database searches with realistic statistics. *Biol. Direct*, **2**, 25.
- Alves, G., Ogurtsov, A. Y., Kwok, S., Wu, W. W., Wang, G., Shen, R. F., and Yu, Y. K. (2008a). Detection of co-eluted peptides using database search methods. *Biol. Direct*, **3**, 27.
- Alves, G., Wu, W. W., Wang, G., Shen, R. F., and Yu, Y. K. (2008b). Enhancing peptide identification confidence by combining search methods. *J. Proteome Res.*, **7**(8), 3102–3113.
- Alves, G., Ogurtsov, A. Y., and Yu, Y. K. (2010). RAId.aPS: MS/MS analysis with multiple scoring functions and spectrum-specific statistics. *PLoS ONE*, **5**(11), e15438.
- Bahrucha-Reid, A. (1960). *Elements of the Theory of Markov Processes and their Applications*. McGraw-Hill.
- Benjamini, Y. and Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, **57**(1), 289–300.
- Elias, J. E. and Gygi, S. P. (2007). Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat. Methods*, **4**(3), 207–214.
- Eng, J. K., McCormack, A. L., and III, J. R. Y. (1994). An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *Journal of the American Society for Mass Spectrometry*, **5**(11), 976–989.
- Feng, J., Naiman, D. Q., and Cooper, B. (2007). Probability-based pattern recognition and statistical framework for randomization: modeling tandem mass spectrum/peptide sequence false match frequencies. *Bioinformatics*, **23**(17), 2210–2217.
- Fenyo, D. and Beavis, R. C. (2003). A method for assessing the statistical significance of mass spectrometry-based protein identifications using general scoring schemes. *Anal. Chem.*, **75**(4), 768–774.
- Fenyo, D., Eriksson, J., and Beavis, R. (2010). Mass spectrometric protein identification using the global proteome machine. *Methods Mol. Biol.*, **673**, 189–202.
- Fisher, R. A. (1932). *Statistical Methods for Research Workers*, vol. II. Oliver and Boyd, Edinburgh.
- Good, I. J. (1955). On the weighted combination of significance tests. *Journal of the Royal Statistical Society Series B (Methodological)*, **17**, 264–265.
- Gupta, N., Bandeira, N., Keich, U., and Pevzner, P. A. (2011). Target-decoy approach and false discovery rate: when things may go wrong. *J. Am. Soc. Mass Spectrom.*, **22**(7), 1111–1120.
- Huang, T., Wang, J., Yu, W., and He, Z. (2012). Protein inference: a review. *Briefings in Bioinformatics*.
- Li, Y. F. and Radivojac, P. (2012). Computational approaches to protein inference in shotgun proteomics. *BMC Bioinformatics*, **13** Suppl 16, S4.
- Mathai, A. (1983). On linear combinations of independent exponential variables. *Communications in Statistics - Theory and Methods*, **12**(6), 625–632.
- McHugh, L. and Arthur, J. W. (2008). Computational methods for protein identification from mass spectrometry data. *PLoS Comput. Biol.*, **4**(2), e12.
- Nesvizhskii, A. I., Keller, A., Kolker, E., and Aebersold, R. (2003). A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.*, **75**(17), 4646–4658.
- Noble, W. S. and MacCoss, M. J. (2012). Computational and statistical analysis of protein mass spectrometry data. *PLoS Comput. Biol.*, **8**(1), e1002296.
- Park, C. Y., Klammer, A. A., Kall, L., MacCoss, M. J., and Noble, W. S. (2008). Rapid and accurate peptide identification from tandem mass spectra. *J. Proteome Res.*, **7**(7), 3022–3027.
- Price, T. S., Lucitt, M. B., Wu, W., Austin, D. J., Pizarro, A., Yocum, A. K., Blair, I. A., FitzGerald, G. A., and Grosser, T. (2007). EBP, a program for protein identification using multiple tandem mass spectrometry datasets. *Mol. Cell Proteomics*, **6**(3), 527–536.
- Robinson, A. B. and Robinson, L. R. (1991). Distribution of glutamine and asparagine residues and their near neighbors in peptides and proteins. *Proc. Natl. Acad. Sci. USA*, **88**, 8880–8884.
- Searle, B. C. (2010). Scaffold: a bioinformatic tool for validating MS/MS-based proteomic studies. *Proteomics*, **10**(6), 1265–1269.

- Segal, M. R. (2008). On E-values for tandem MS scoring schemes. *Bioinformatics*, **24**(14), 1652–1653.
- Serang, O. and Noble, W. (2012). A review of statistical methods for protein identification using tandem mass spectrometry. *Stat Interface*, **5**(1), 3–20.
- Serang, O., Paulo, J., Steen, H., and Steen, J. A. (2013). A non-parametric cutout index for robust evaluation of identified proteins. *Mol. Cell Proteomics*, **12**(3), 807–812.
- Shteynberg, D., Deutsch, E. W., Lam, H., Eng, J. K., Sun, Z., Tasman, N., Mendoza, L., Moritz, R. L., Aebersold, R., and Nesvizhskii, A. I. (2011). iProphet: multi-level integrative analysis of shotgun proteomic data improves peptide and protein identification rates and error estimates. *Mol. Cell Proteomics*, **10**(12), M111.007690.
- Sorić, B. (1989). Statistical “discoveries” and effect-size estimation. *Journal of the American Statistical Association*, **84**(406), 608–610.
- Spirin, V., Shpunt, A., Seebacher, J., Gentzel, M., Shevchenko, A., Gygi, S., and Sunyaev, S. (2011). Assigning spectrum-specific P-values to protein identifications by mass spectrometry. *Bioinformatics*, **27**(8), 1128–1134.
- Wang, G., Wu, W. W., Zhang, Z., Masilamani, S., and Shen, R. F. (2009). Decoy methods for assessing false positives and false discovery rates in shotgun proteomics. *Anal. Chem.*, **81**(1), 146–159.
- Whitlock, M. C. (2005). Combining probability from independent tests: the weighted Z-method is superior to Fisher’s approach. *J. Evol. Biol.*, **18**(5), 1368–1373.
- Yang, X., Dondeti, V., Dezube, R., Maynard, D. M., Geer, L. Y., Epstein, J., Chen, X., Markey, S. P., and Kowalak, J. A. (2004). DBParser: web-based software for shotgun proteomic data analyses. *J. Proteome Res.*, **3**(5), 1002–1008.
- Yu, Y. K., Gertz, E. M., Agarwala, R., Schaffer, A. A., and Altschul, S. F. (2006). Retrieval accuracy, statistical significance and compositional similarity in protein sequence database searches. *Nucleic Acids Res.*, **34**(20), 5966–5973.
- Zhang, W. and Chait, B. T. (2000). ProFound: an expert system for protein identification using mass spectrometric peptide mapping information. *Anal. Chem.*, **72**(11), 2482–2489.
- Zhang, Y., Fonslow, B. R., Shan, B., Baek, M. C., and Yates, J. R. (2013). Protein analysis by shotgun/bottom-up proteomics. *Chem. Rev.*, **113**(4), 2343–2394.

SUPPLEMENTARY INFORMATION

Example application of formula (3) of the main text

To illustrate the use of the formula used to compute a protein P -value, let us consider the following toy example. Let protein Π have six peptide evidences $\pi_1, \pi_2, \dots, \pi_6$ that falls into three groups $\{\pi_1\}$, $\{\pi_2, \pi_3\}$, and $\{\pi_4, \pi_5, \pi_6\}$, respectively with weights 1, $1/3$, $1/2$. This means that peptide π_1 is a subsequence of protein Π only, peptides π_2 and π_3 are subsequences of three proteins (Π and two others), and peptides π_4, π_5 , and π_6 are subsequences of two proteins (Π and another one). Let the peptide E -values of these six evidence peptides be e_1, e_2, \dots, e_6 , all less than one. Under our approximating database P -value by E -value, this means that the evidence peptides have their respective database P -values e_1, e_2, \dots, e_6 . From the information above we know that $m = 3$, $n_1 = 1$, $n_2 = 2$, $n_3 = 3$, $w_1 = 1$, $w_2 = 1/3$, $w_3 = 1/2$, $r_1 = 1/w_1 = 1$, $r_2 = 1/w_2 = 3$, and $r_3 = 1/w_3 = 2$.

To use formula (3) in the manuscript to compute a protein P -value, we first need the quantity τ given by eq. (1) in the main text. In the current case— $m = 3$, $n_1 = 1$, $n_2 = 2$, $n_3 = 3$ and with peptides database P -values e_1, e_2, \dots, e_6 — the quantity τ can be written as

$$\tau = \prod_{k=1}^3 \left[\prod_{j=1}^{n_k} e_{k;j} \right]^{w_k} = e_1^{w_1} e_2^{w_2} e_3^{w_2} e_4^{w_3} e_5^{w_3} e_6^{w_3}.$$

And the protein P -value is given by

$$F(\tau) = \left[\prod_{l=1}^3 r_l^{n_l} \right] \sum_{k=1}^3 \sum_{\mathcal{G}(k)} \left\{ \frac{1}{r_k^{g_k+1}} H(-r_k \ln \tau, g_k) \times \left(\prod_{j=1, j \neq k}^3 \frac{(n_j - 1 + g_j)!}{(n_j - 1)! g_j!} \frac{(-1)^{g_j}}{(r_j - r_k)^{n_j + g_j}} \right) \right\}, \quad (6)$$

where $H(-r_k \ln \tau, g_k)$ is given by

$$H(-r_k \ln \tau, g_k) = \exp(r_k \ln \tau) \sum_{k=0}^{g_k} \frac{(-r_k \ln \tau)^k}{k!}.$$

Remember that $\sum_{\mathcal{G}(k)}$ enumerates each set of nonnegative integers $\{g_1, g_2, \dots, g_m\}$ that satisfies the k -dependent constraint $\sum_{i=1}^m g_i = n_k - 1$, it is thus possible to replace the $\sum_{\mathcal{G}(k)}$ in eq. (6) by an m -dimensional summation with an explicit constraint. Specifically, we can rewrite the sum over set as

$$F(\tau) = \left[\prod_{l=1}^3 r_l^{n_l} \right] \sum_{k=1}^3 \sum_{g_1=0}^{n_k-1} \sum_{g_2=0}^{n_k-1} \sum_{g_3=0}^{n_k-1} \delta_{g_1+g_2+g_3, n_k-1} \left\{ \frac{1}{r_k^{g_k+1}} H(-r_k \ln \tau, g_k) \times \right. \\ \left. \times \left(\prod_{j=1, j \neq k}^3 \frac{(n_j - 1 + g_j)!}{(n_j - 1)! g_j!} \frac{(-1)^{g_j}}{(r_j - r_k)^{n_j + g_j}} \right) \right\}. \quad (7)$$

where the Kronecker delta function $\delta_{n,n'}$ takes value one if $n = n'$ but zero otherwise.

The first product on the right hand side of eq. (7) is equal to

$$\left[\prod_{l=1}^3 r_l^{n_l} \right] = (r_1)^1 \cdot (r_2)^2 \cdot (r_3)^3 = 1^1 \cdot 3^2 \cdot 2^3.$$

After this overall factor is obtained, the main task is to evaluate the summation over k , which ranges from $k = 1$ to $k = 3$. For each k , we are only interested in the non-negative integral g_i s that satisfy the k -dependent constraint $\sum_{i=1}^3 g_i = n_k - 1$. When $k = 1$ we have $n_1 = 1$ and the constrained summation

$$\sum_{g_1=0}^0 \sum_{g_2=0}^0 \sum_{g_3=0}^0 \delta_{g_1+g_2+g_3, 0}$$

only allows one valid $\{g_1, g_2, g_3\}$ set, namely, $\{0,0,0\}$. For $k = 2$ we have $n_2 = 2$ and the constrained summation

$$\sum_{g_1=0}^1 \sum_{g_2=0}^1 \sum_{g_3=0}^1 \delta_{g_1+g_2+g_3, 1}$$

allows three sets of valid $\{g_1, g_2, g_3\}$, namely, $\{1,0,0\}$, $\{0,1,0\}$ and $\{0,0,1\}$. For $k = 3$ we have $n_3 = 3$ and the constrained summation

$$\sum_{g_1=0}^2 \sum_{g_2=0}^2 \sum_{g_3=0}^2 \delta_{g_1+g_2+g_3, 2}$$

allows six sets of valid $\{g_1, g_2, g_3\}$, namely, $\{1,1,0\}$, $\{1,0,1\}$, $\{0,1,1\}$, $\{2,0,0\}$, $\{0,2,0\}$ and $\{0,0,2\}$. Each valid set of $\{g_1, g_2, g_3\}$ must be substituted into the summand (inside the pair of curly braces) of eq. (7) to yield its respective contribution for the P -value.

Supplementary Tables

Table S1. MS/MS data group 1. The corresponding spectral datasets are produced by using Sigma49 (a protein standard mixture composed of 49 known human proteins), and are downloaded from the National Center for Biotechnology Information (Peptidome database) at <ftp://ftp.ncbi.nih.gov/pub/peptidome/studies/PSEnnn/PSE108>. The column heading SN represents sample index, the abbreviation CGL stands for chromatography gradient length, and the column heading n_s stands for the number of spectra acquired. The rest of the column headings are self-explanatory.

SN	Sample Load	Instrument	CGL(minutes)	n_s	File Name
1	5 fmol	LTQ Orbitrap	45	1,531	PSM1027_07FEB15_ABRF_FT_5a.mzXML
2	5 fmol	LTQ Orbitrap	45	1,902	PSM1028_07FEB15_ABRF_FT_5b.mzXML
3	5 fmol	LTQ Orbitrap	45	2,014	PSM1029_07FEB15_ABRF_FT_5c.mzXML
4	10 fmol	LTQ Orbitrap	45	2,026	PSM1027_07FEB15_ABRF_FT_10a.mzXML
5	10 fmol	LTQ Orbitrap	45	2,125	PSM1028_07FEB15_ABRF_FT_10b.mzXML
6	10 fmol	LTQ Orbitrap	45	2,253	PSM1029_07FEB15_ABRF_FT_10c.mzXML
7	25 fmol	LTQ Orbitrap	45	2,772	PSM1027_07FEB15_ABRF_FT_25a.mzXML
8	25 fmol	LTQ Orbitrap	45	2,669	PSM1028_07FEB15_ABRF_FT_25b.mzXML
9	25 fmol	LTQ Orbitrap	45	2,504	PSM1029_07FEB15_ABRF_FT_25c.mzXML
10	50 fmol	LTQ Orbitrap	45	3,259	PSM1027_07FEB15_ABRF_FT_50a.mzXML
11	50 fmol	LTQ Orbitrap	45	3,406	PSM1028_07FEB15_ABRF_FT_50b.mzXML
12	50 fmol	LTQ Orbitrap	45	2,993	PSM1029_07FEB15_ABRF_FT_50c.mzXML
13	100 fmol	LTQ Orbitrap	45	3,629	PSM1027_07FEB15_ABRF_FT_100a.mzXML
14	100 fmol	LTQ Orbitrap	45	3,622	PSM1028_07FEB15_ABRF_FT_100b.mzXML
15	100 fmol	LTQ Orbitrap	45	3,592	PSM1029_07FEB15_ABRF_FT_100c.mzXML

Table S2. MS/MS data group 2. The corresponding spectral datasets are produced by using a complex protein mixture of *Escherichia coli K-12* whole cell lysate, and are downloaded from the Pacific Northwest National Laboratory at <http://omics.pnl.gov/>.

SN	Instrument	CGL(minutes)	n_s	File Name
16	LTQ Orbitrap	100	18,573	Ecoli432_R1_rr_18Dec09_Falcon_09-09-14.mzXML
17	LTQ Orbitrap	100	18,585	Ecoli432_R2_7Dec09_Falcon_09-09-15.mzXML
18	LTQ Orbitrap	100	18,669	Ecoli432_R3_7Dec09_Falcon_09-09-16.mzXML
19	LTQ Orbitrap	100	18,585	Ecoli432_R4_15Dec09_Falcon_09-09-16.mzXML
20	LTQ Orbitrap	100	18,650	Ecoli433_R1_7Dec09_Falcon_09-09-14.mzXML
21	LTQ Orbitrap	100	18,763	Ecoli433_R2_7Dec09_Falcon_09-09-15.mzXML
22	LTQ Orbitrap	100	18,770	Ecoli433_R4_13Dec09_Falcon_09-09-16.mzXML
23	LTQ Orbitrap	100	18,488	Ecoli434_R1_7Dec09_Falcon_09-09-14.mzXML
24	LTQ Orbitrap	100	18,923	Ecoli434_R2_7Dec09_Falcon_09-09-15.mzXML
25	LTQ Orbitrap	100	19,010	Ecoli434_R3_7Dec09_Falcon_09-09-16.mzXML
26	LTQ Orbitrap	100	18,737	Ecoli434_R4_13Dec09_Falcon_09-09-16.mzXML

Table S3. MS/MS data group 3. The corresponding spectral datasets are produced by using a complex protein mixture of *Escherichia coli* whole cell lysate prepared in house.

SN	Instrument	CGL(minutes)	n_s	File Name
27	Orbitrap Elite	90	24,280	E_L_2.mzML
28	Orbitrap Elite	90	22,435	E_M_2.mzML
29	Orbitrap Elite	90	23,875	E_H_2.mzML
30	Orbitrap Elite	90	18,573	E_S_2.mzML

Table S4. MS/MS data group 4. The corresponding spectral datasets are produced by using SDS-PAGE protein fractionation extraction of human lung cells, and are downloaded from PeptideAtlas database at <ftp://ftp.peptideatlas.org/pub/PeptideAtlas/Repository/PAe001771>.

SN	Instrument	CGL(minutes)	n_s	File Name
31-63	LTQ Orbitrap	120	340,861	Roche_human_lung_001.mzML - Roche_human_lung_033.mzML

Supplementary Figures

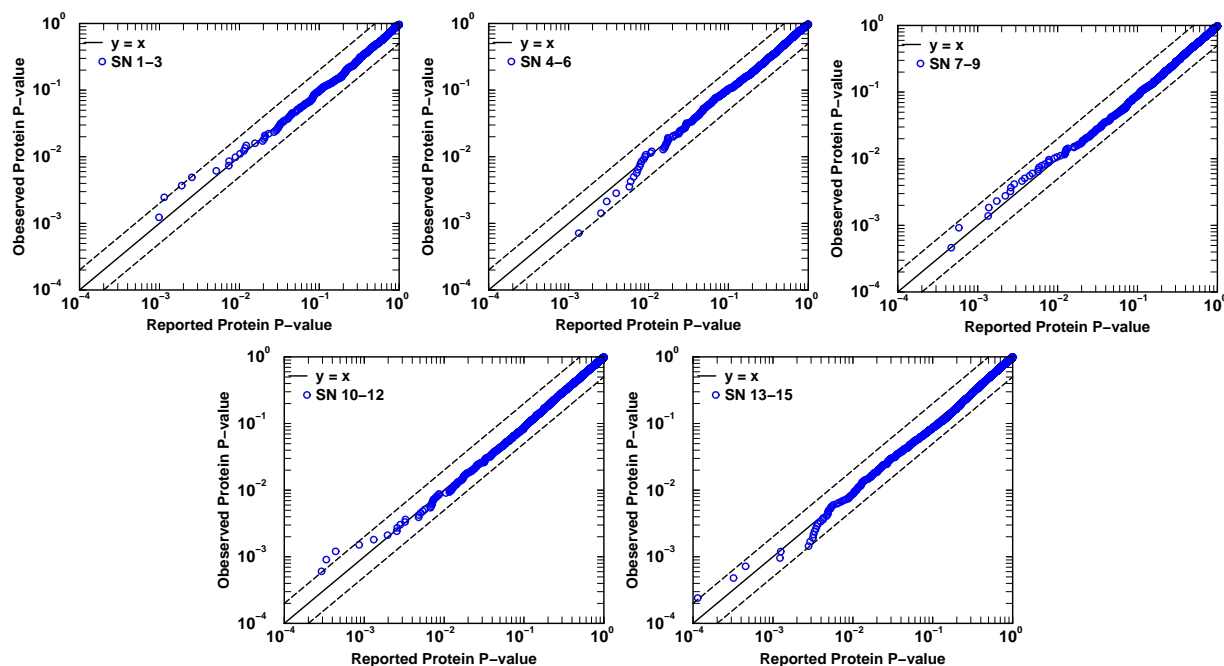


Fig. S1. Accuracy assessment of the protein P -value reported by the proposed method using data group 1 and RAId_DbS's peptide E -values. All spectra in data group 1 are used to search the *Homo sapiens* database with precursor-ion mass tolerance ± 0.033 Da., product-ion mass tolerance ± 0.8 Da. and up-to-2 missed cleavage sites allowed. The accuracy of the reported protein P -value is evaluated by plotting it versus the observed protein P -value, see main text for details. The closer the above curves are to the $y = x$ line the more accurate are the reported protein P -values. The two dash lines, $y = 2x$ and $y = 0.5x$, are provided as visual guides regarding how close/off the reported protein P -values are to the $y = x$ line.

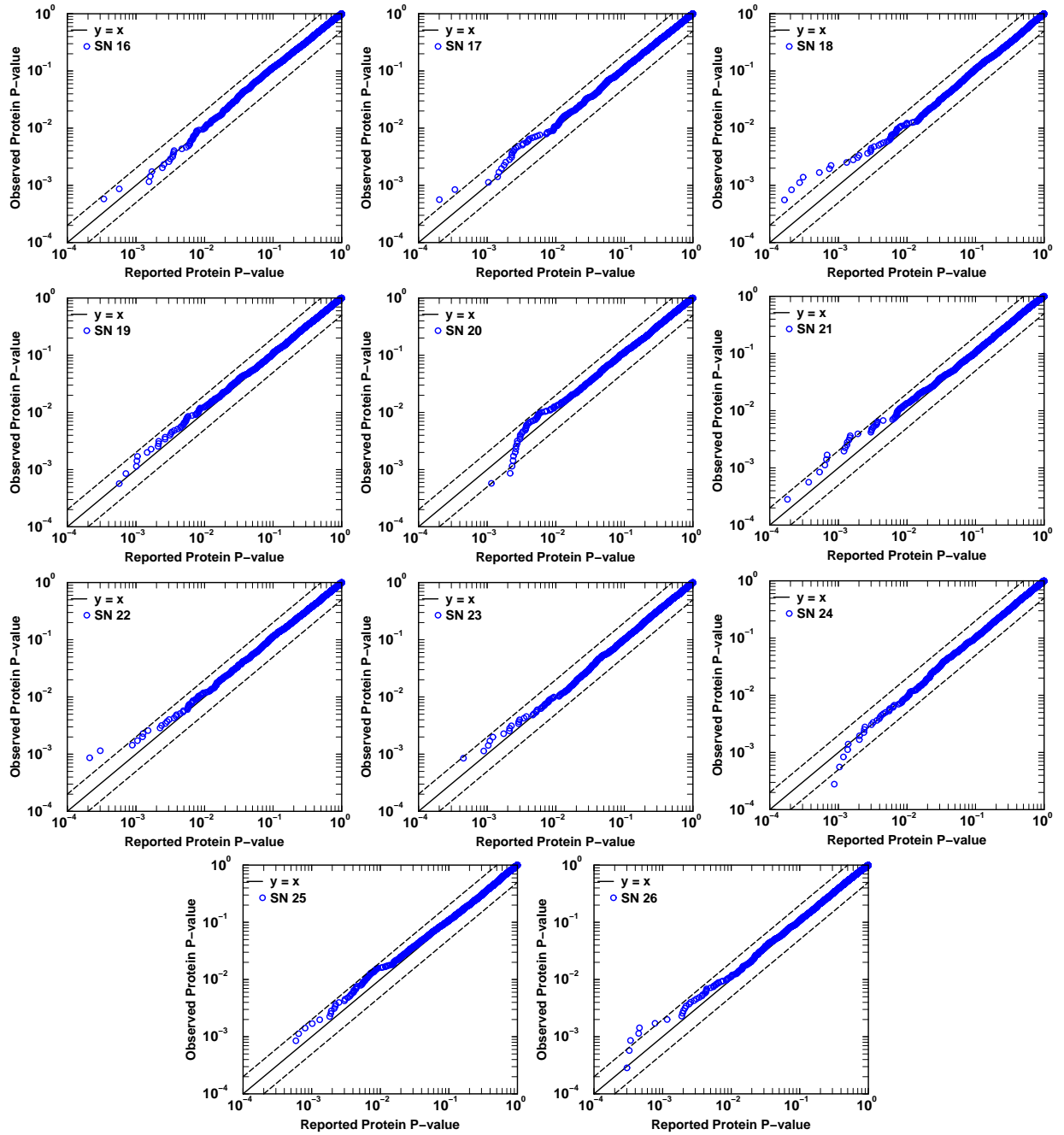


Fig. S2. Accuracy assessment of the protein P -value reported by the proposed method using data group 2 and RAId.DbS's peptide E -values. All spectra in data group 2 are used to search the *Escherichia coli* database with precursor-ion mass tolerance ± 0.033 Da., product-ion mass tolerance ± 0.033 Da. and up-to-2 missed cleavage sites allowed. The accuracy of the reported protein P -value is evaluated by plotting it versus the observed protein P -value, see main text for details. The closer the above curves are to the $y = x$ line the more accurate are the reported protein P -values. The two dash lines, $y = 2x$ and $y = 0.5x$, are provided as visual guides regarding how close/off the reported protein P -values are to the $y = x$ line.

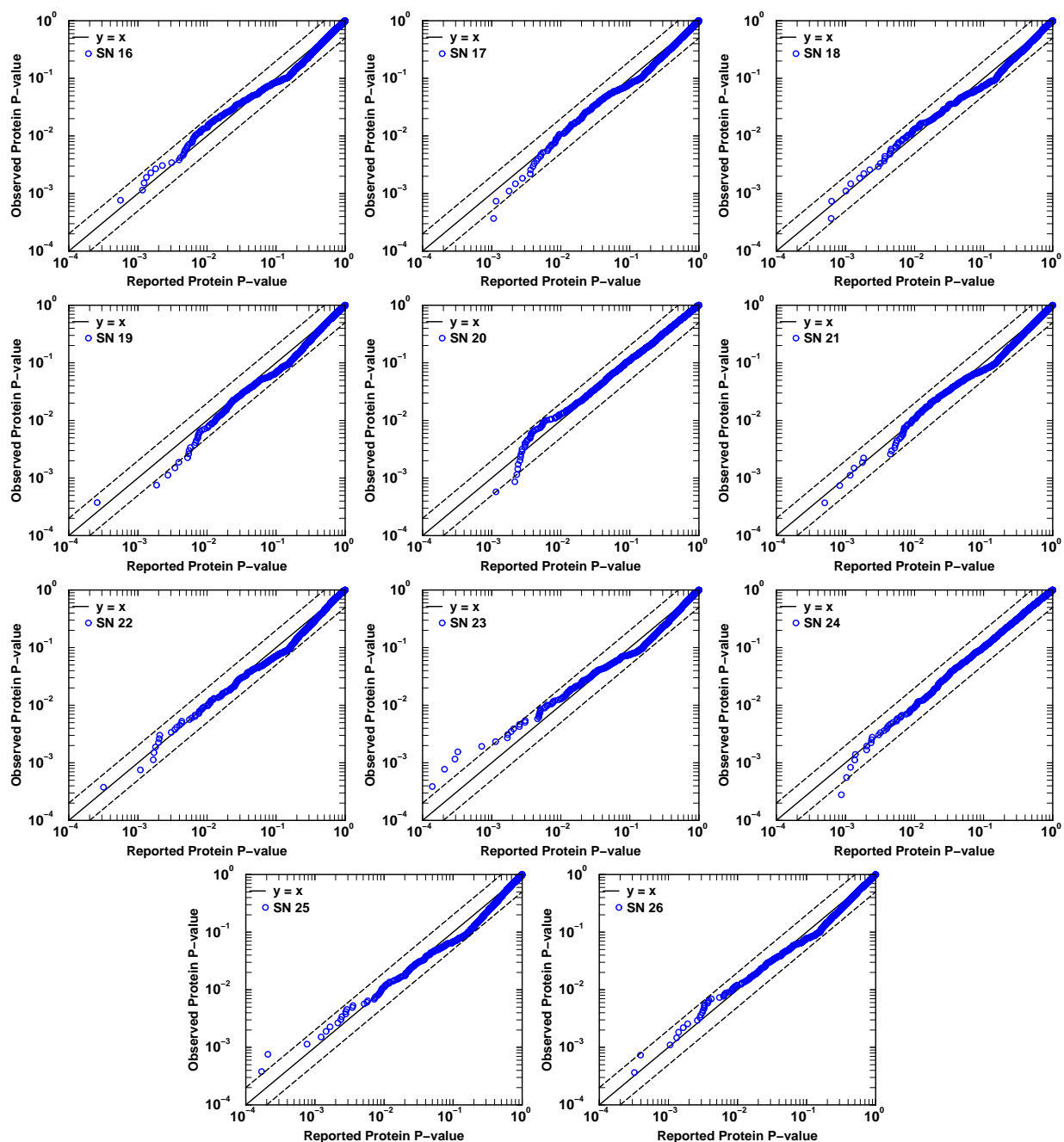


Fig. S3. Accuracy assessment of the reported protein P -value by the proposed method when the mass tolerances are very small. The protein P -values are obtained by using RAId_DbS's peptide E -values. All spectra in data group 2 are used to search the *Escherichia coli* database with precursor-ion mass tolerance ± 0.0033 Da., product-ion mass tolerance ± 0.0033 Da. and up-to-2 missed cleavage sites allowed. The accuracy of the reported protein P -value is evaluated by plotting it versus the observed protein P -value, see main text for details. The closer the above curves are to the $y = x$ line the more accurate are the reported protein P -values. The two dash lines, $y = 2x$ and $y = 0.5x$, are provided as visual guides regarding how close/off the reported protein P -values are to the $y = x$ line.

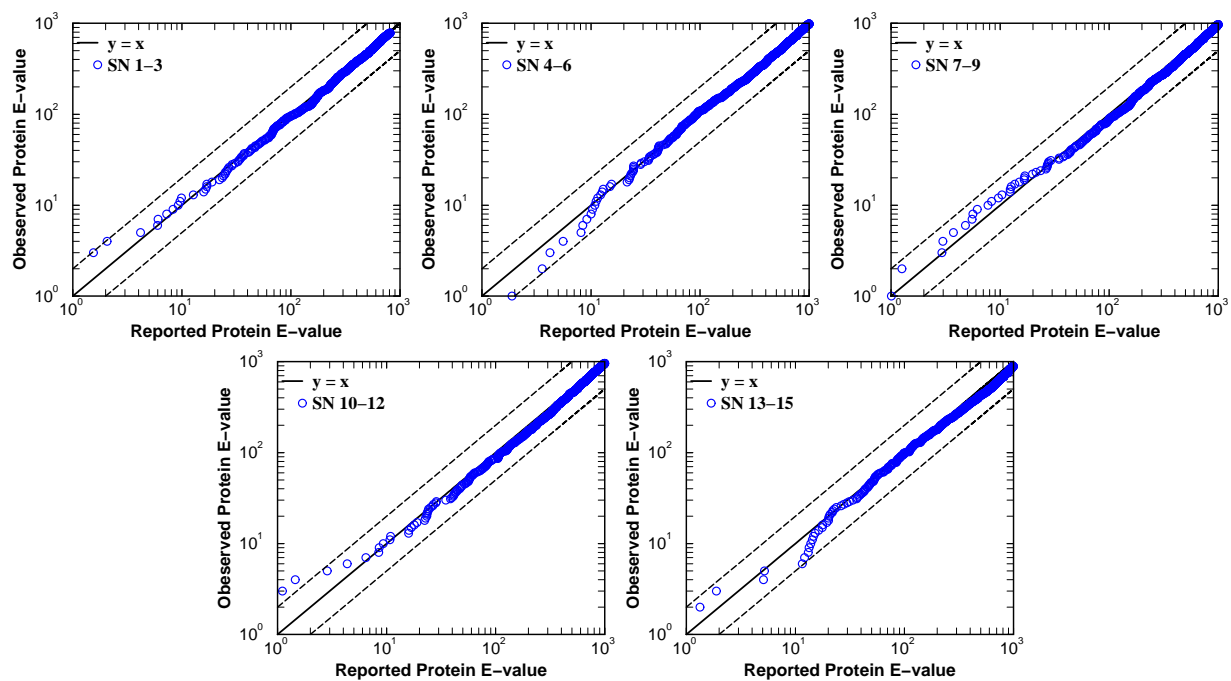


Fig. S4. Accuracy assessment of the protein E -value reported by the proposed method using data group 1 and RAId_DbS's peptide E -values. All spectra in data group 1 are used to search the *Homo sapiens* database with precursor-ion mass tolerance ± 0.033 Da., product-ion mass tolerance ± 0.8 Da. and up-to-2 missed cleavage sites allowed. The accuracy of the reported protein P -value is evaluated by plotting it versus the observed protein P -value, see main text for details. The closer the above curves are to the $y = x$ line the more accurate are the reported protein E -values. The two dash lines, $y = 2x$ and $y = 0.5x$, are provided as visual guides regarding how close/off the reported protein E -values are to the $y = x$ line.

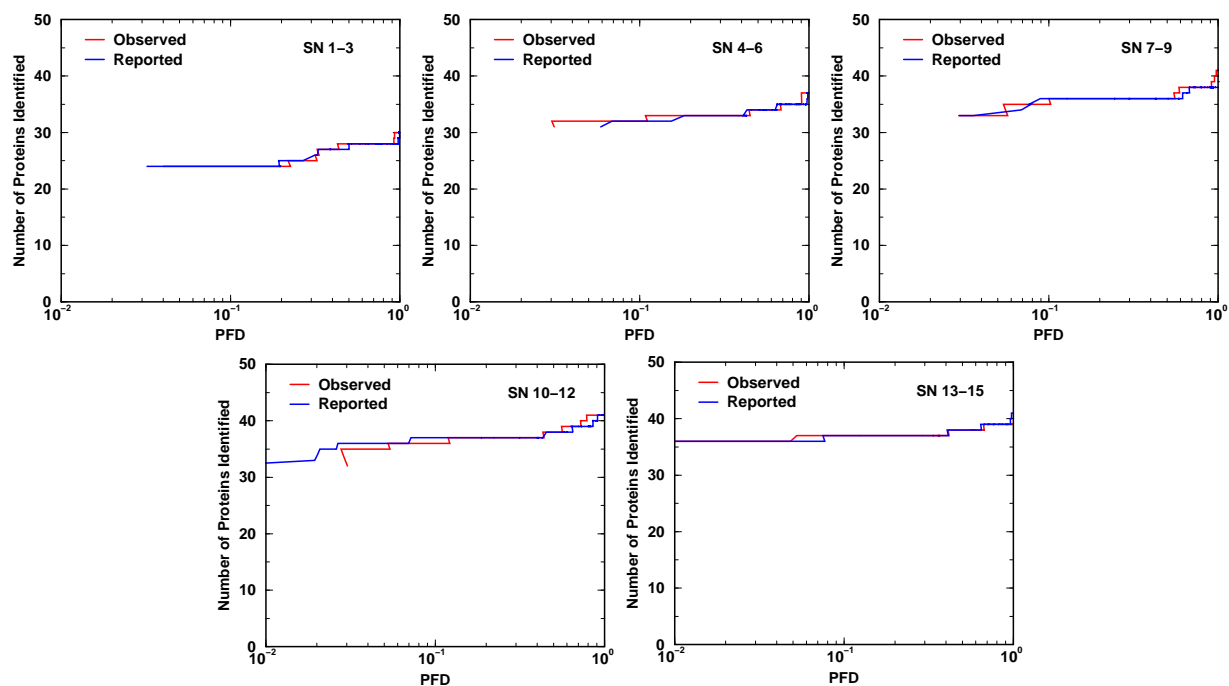


Fig. S5. Accuracy assessment of reported protein PFD using data group 1 and RAId_DbS's peptide E -values. All spectra in data group 1 (MS/MS spectra from SN 1-15) are used to search the *Homo sapiens* database with precursor-ion mass tolerance ± 0.033 Da., product-ion mass tolerance ± 0.8 Da. and up-to-2 missed cleavage sites allowed. The reported PFD is computed by using the reported protein E -value in Sorić's formula, while the observed PFD is obtained from the ratio of the number of false discoveries (false positives) to the total number of discoveries (true positives + false positives) at a given E -value cutoff. The closer the observed and reported curves are to each other the better.

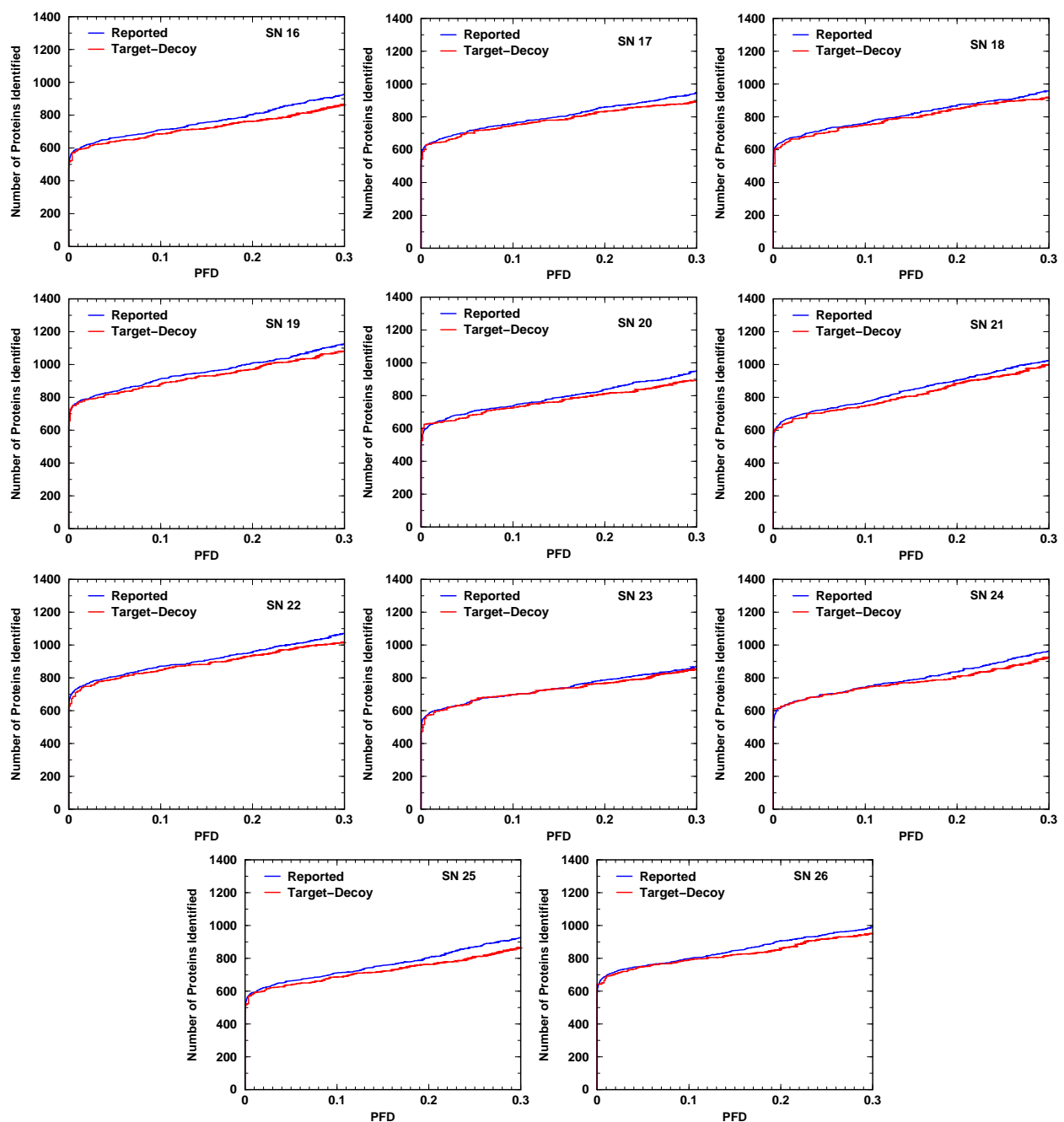


Fig. S6. The agreement between the *E*-value based PFD and the target-decoy based PFD when the peptide *E*-values are from RAId.DbS. All spectra in data group 2 (MS/MS spectra SN16-26) are used to search the *Escherichia coli* database with precursor-ion mass tolerance ± 0.033 Da., product-ion mass tolerance ± 0.033 Da. and up-to-2 missed cleavage sites allowed. The *E*-value based PFD is computed by using the reported protein *E*-value in Sorić's formula, while the target-decoy based PFD is obtained from the ratio of the number of identified decoy proteins to the total number of identified proteins (target + decoy) for a given *E*-value cutoff. Within each panel, the closer the two curves are to each other the better.

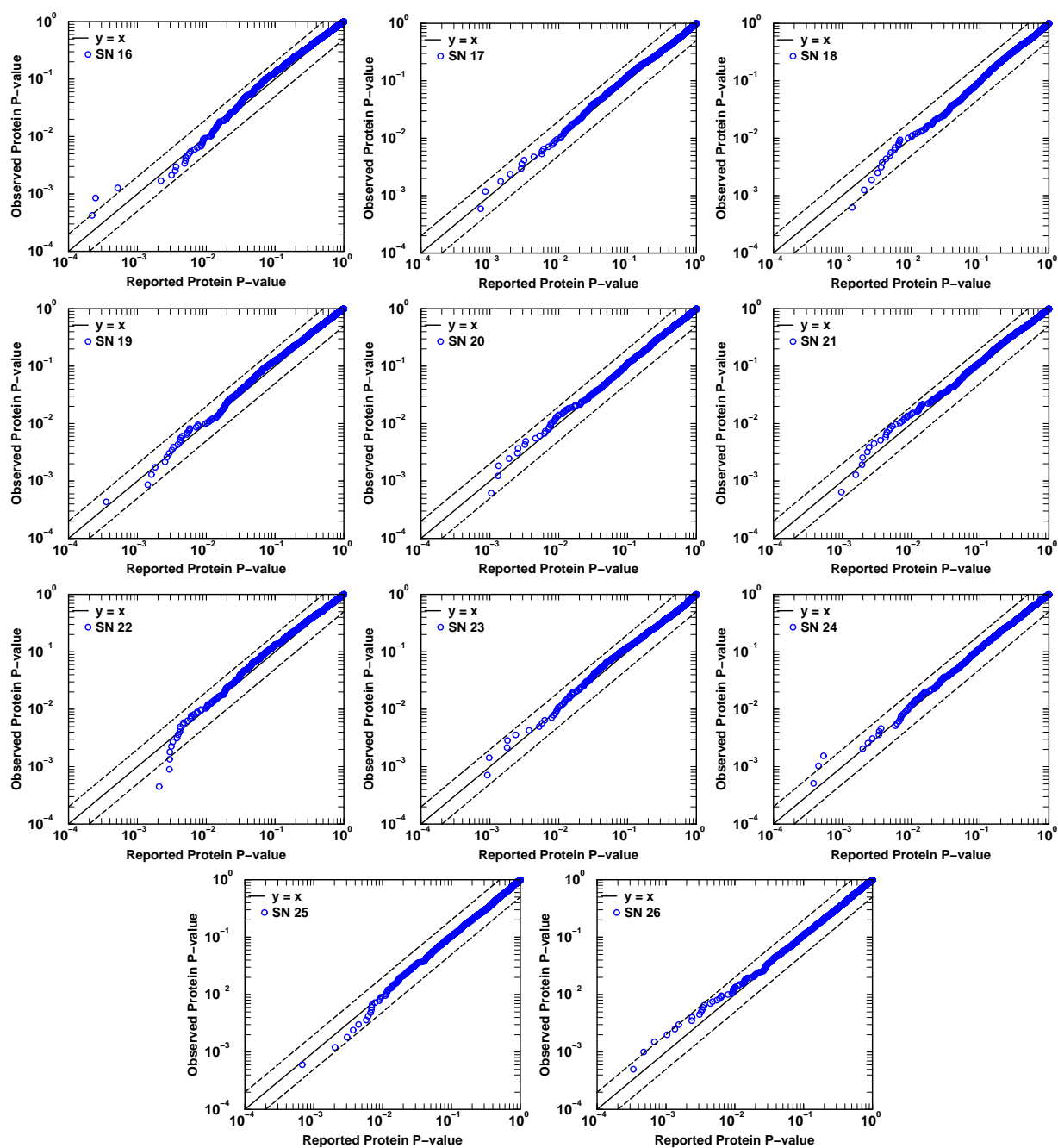


Fig. S7. Accuracy assessment of the protein P -value using data group 2 and RAID.aPS's peptide E -values when the selected scoring function is XCorr. All spectra in data group 2 are used to search the *Escherichia coli* database with precursor-ion mass tolerance ± 0.033 Da., product-ion mass tolerance ± 0.033 Da. and up-to-2 missed cleavage sites allowed. The accuracy of the reported protein P -value is evaluated by plotting it versus the observed protein P -value, see main text for details. The closer the above curves are to the $y = x$ line the more accurate are the reported protein P -values. The two dash lines, $y = 2x$ and $y = 0.5x$, are provided as visual guides regarding how close/off the reported protein P -values are to the $y = x$ line.

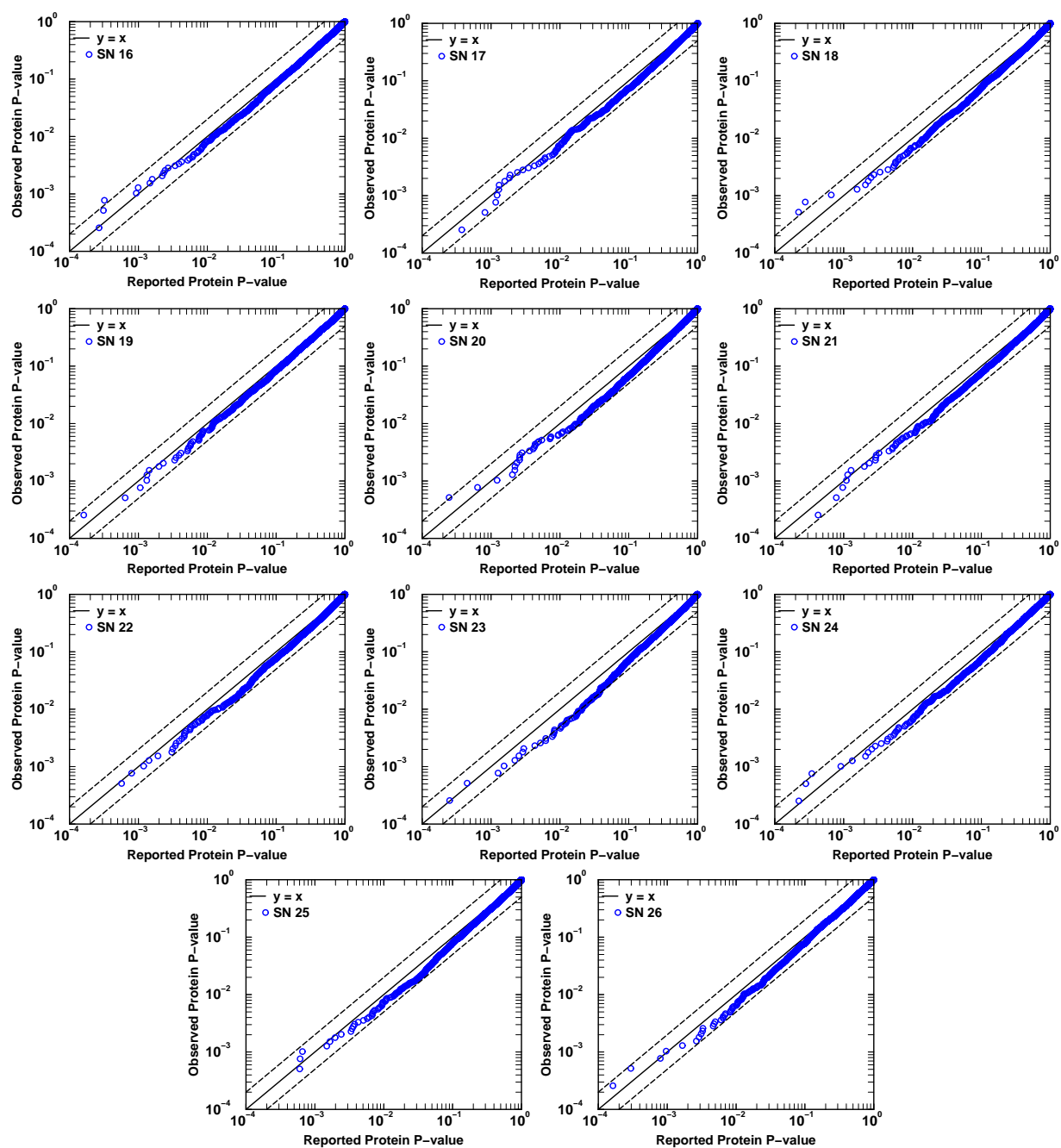


Fig. S8. Accuracy assessment of the protein P -value using data group 2 and RAId_aPS's peptide E -values when the selected scoring function is **Hyperscore**. All spectra in data group 2 are used to search the *Escherichia coli* database with precursor-ion mass tolerance ± 0.033 Da., product-ion mass tolerance ± 0.033 Da. and up-to-2 missed cleavage sites allowed. The accuracy of the reported protein P -value is evaluated by plotting it versus the observed protein P -value, see main text for details. The closer the above curves are to the $y = x$ line the more accurate are the reported protein P -values. The two dash lines, $y = 2x$ and $y = 0.5x$, are provided as visual guides regarding how close/off the reported protein P -values are to the $y = x$ line.

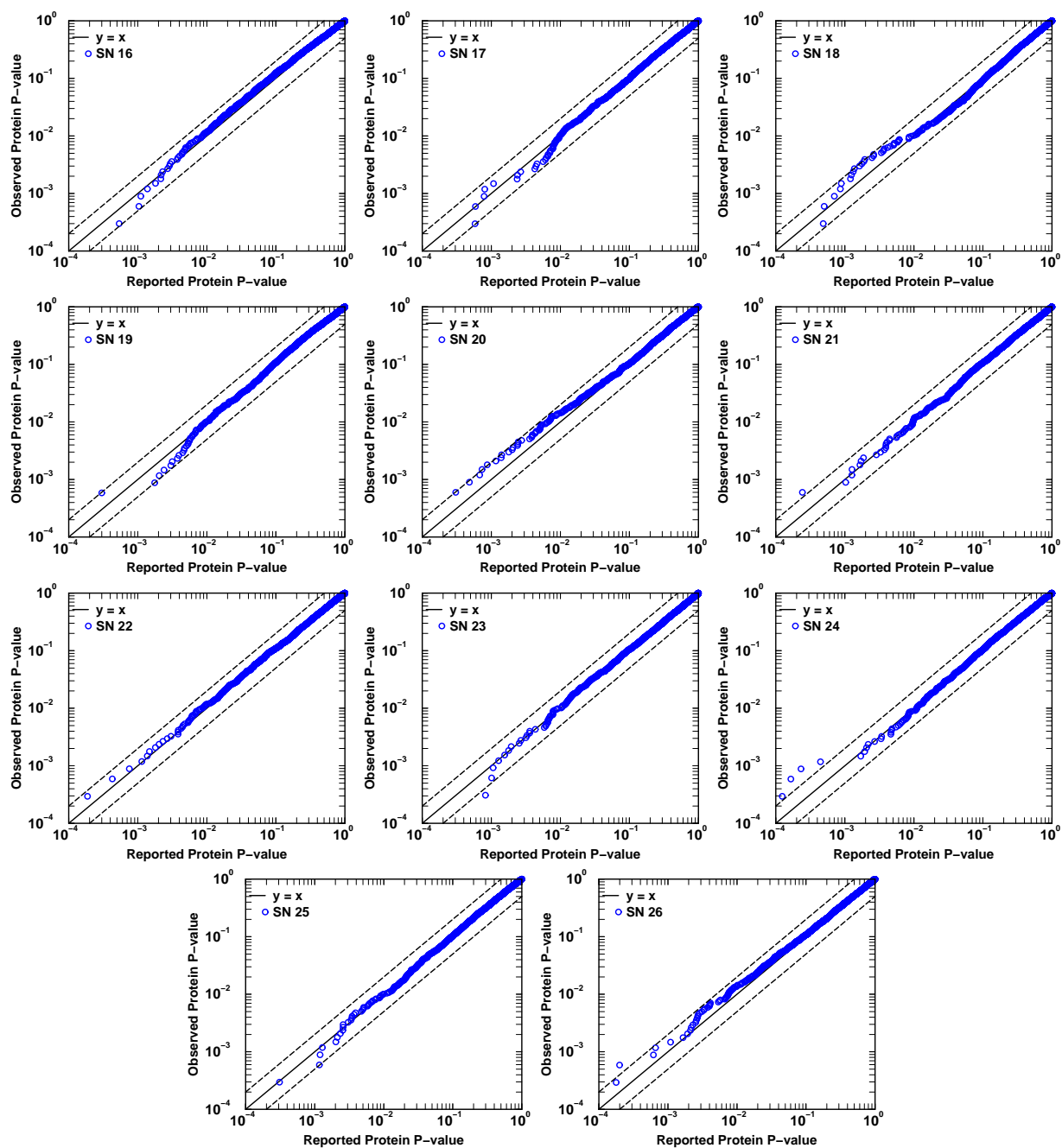


Fig. S9. Accuracy assessment of the protein P -value using data group 2 and RAId.aPS's peptide E -values when the selected scoring function is **Kscore**. All spectra in data group 2 are used to search the *Escherichia coli* database with precursor-ion mass tolerance ± 0.033 Da., product-ion mass tolerance ± 0.033 Da. and up-to-2 missed cleavage sites allowed. The accuracy of the reported protein P -value is evaluated by plotting it versus the observed protein P -value, see main text for details. The closer the above curves are to the $y = x$ line the more accurate are the reported protein P -values. The two dash lines, $y = 2x$ and $y = 0.5x$, are provided as visual guides regarding how close/off the reported protein P -values are to the $y = x$ line.

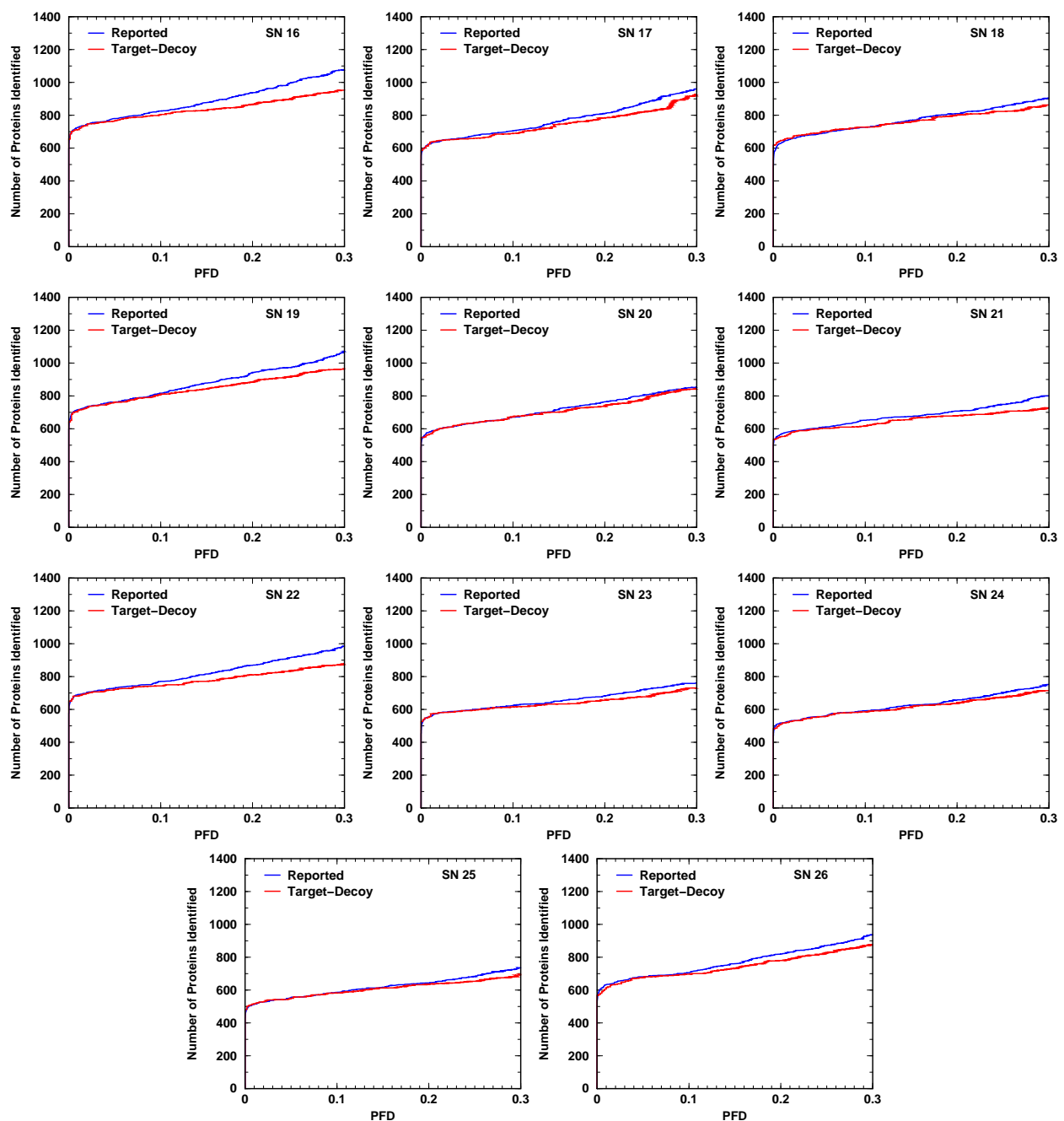


Fig. S10. The agreement between the *E*-value based PFD and the target-decoy based PFD when the peptide *E*-values are from RAId_aPS(XCorr). All spectra in data group 2 (MS/MS spectra SN16-26) are used to search the *Escherichia coli* database with precursor-ion mass tolerance ± 0.033 Da., product-ion mass tolerance ± 0.033 Da. and up-to-2 missed cleavage sites allowed. The *E*-value based PFD is computed by using the reported protein *E*-value in Sorić's formula, while the target-decoy based PFD is obtained from the ratio of the number of identified decoy proteins to the total number of identified proteins (target + decoy) for a given *E*-value cutoff. Within each panel, the closer the two curves are to each other the better.

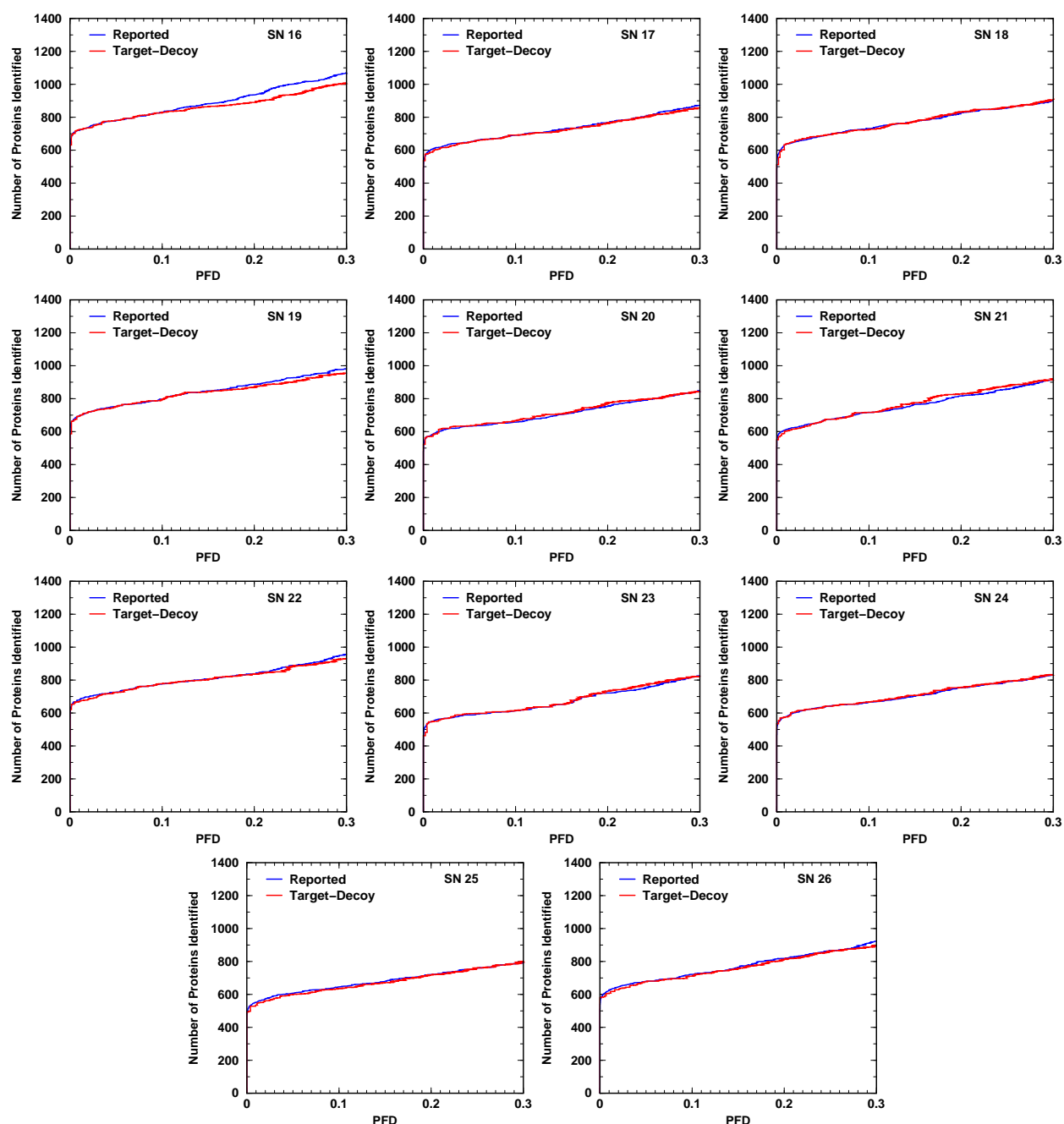


Fig. S11. The agreement between the E -value based PFD and the target-decoy based PFD when the peptide E -values are from **RAIdaPS(Hyperscore)**. All spectra in data group 2 (MS/MS spectra SN16-26) are used to search the *Escherichia coli* database with precursor-ion mass tolerance ± 0.033 Da., product-ion mass tolerance ± 0.033 Da. and up-to-2 missed cleavage sites allowed. The E -value based PFD is computed by using the reported protein E -value in Sorić's formula, while the target-decoy based PFD is obtained from the ratio of the number of identified decoy proteins to the total number of identified proteins (target + decoy) for a given E -value cutoff. Within each panel, the closer the two curves are to each other the better.

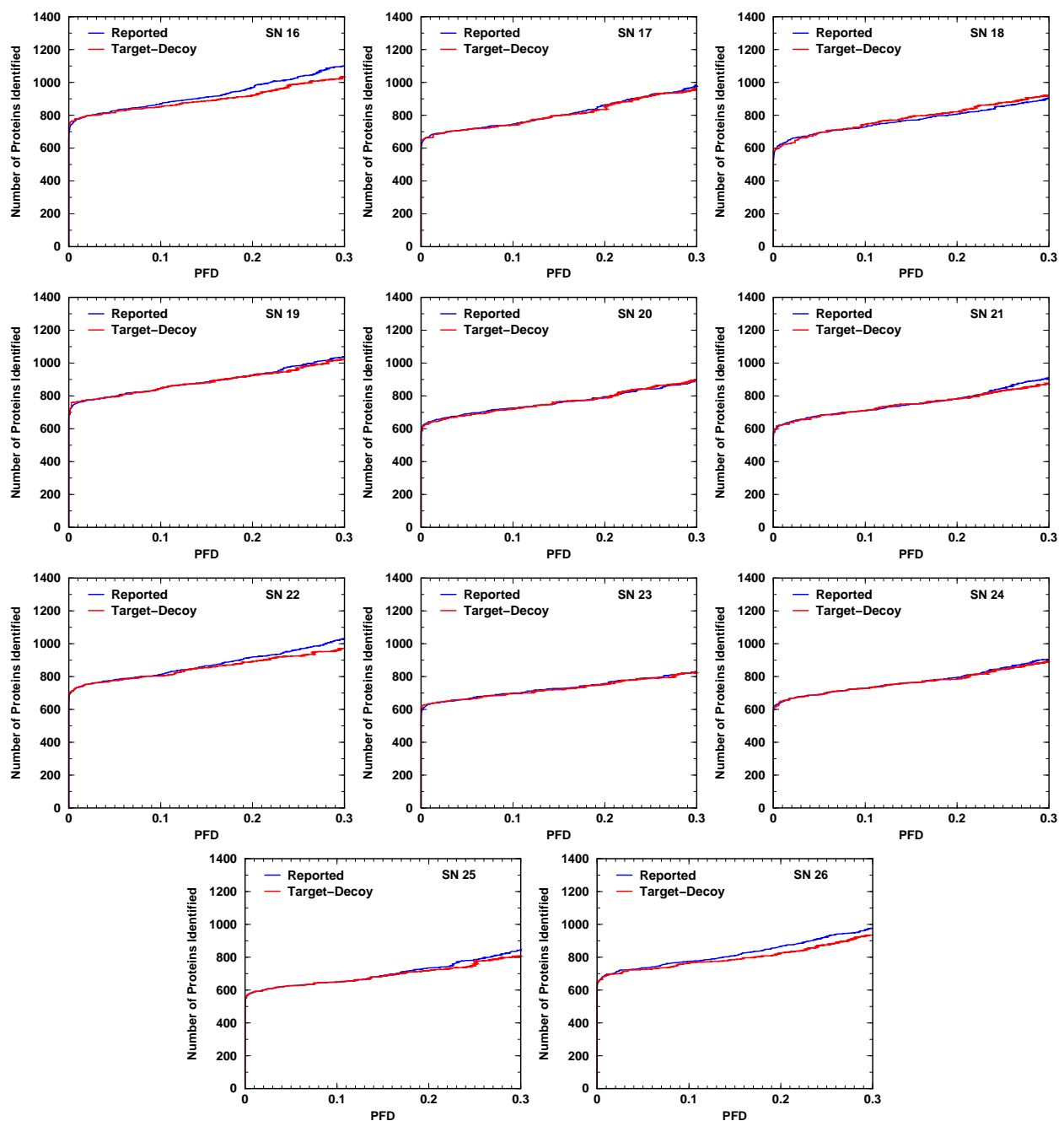


Fig. S12. The agreement between the *E*-value based PFD and the target-decoy based PFD when the peptide *E*-values are from RAId_aPS(Kscore). All spectra in data group 2 (MS/MS spectra SN16-26) are used to search the *Escherichia coli* database with precursor-ion mass tolerance ± 0.033 Da., product-ion mass tolerance ± 0.033 Da. and up-to-2 missed cleavage sites allowed. The *E*-value based PFD is computed by using the reported protein *E*-value in Sorić's formula, while the target-decoy based PFD is obtained from the ratio of the number of identified decoy proteins to the total number of identified proteins (target + decoy) for a given *E*-value cutoff. Within each panel, the closer the two curves are to each other the better.